

Functional Redundancy of CXCR3/CXCL10 Signaling in the Recruitment of Diabetogenic Cytotoxic T Lymphocytes to Pancreatic Islets in a Virally Induced Autoimmune Diabetes Model

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Cytotoxic T lymphocytes (CTLs) constitute a major effector population in pancreatic islets from patients suffering from type 1 diabetes (T1D) and thus represent attractive targets for intervention. Some studies have suggested that blocking the interaction between the chemokine CXCL10 and its receptor CXCR3 on activated CTLs potentially inhibits their recruitment and prevents β -cell death. Since recent studies on human pancreata from T1D patients have indicated that both ligand and receptor are abundantly present, we reevaluated whether their interaction constitutes a pivotal node within the chemokine network associated with T1D. Our present data in a viral mouse model challenge the notion that specific blockade of the CXCL10/CXCR3 chemokine axis halts T1D onset and progression. *Diabetes* 62:2492–2499, 2013

The mechanisms governing autoreactive T-cell homing to pancreatic islets in type 1 diabetes (T1D) are poorly characterized, which in turn has impeded the rational design of therapies at this crucial intersection. Interest in targeting chemokines was sparked by a study that identified β -cells as a key source of CXCL10 in the viral rat insulin promoter (RIP)-lymphocytic choriomeningitis virus (LCMV) diabetes model, which in turn would serve to attract CXCR3-expressing T cells (1). In CXCR3-deficient mice, diabetes onset was markedly delayed. It was subsequently reported in the same model that among CXCR3 ligands, which include CXCL9, -10, and -11, only CXCL10 exerted dominant effects on T-cell recruitment (2).

Several other reports, however, at least partially contradict the idea of CXCL10-mediated attraction of CXCR3-expressing T cells to pancreatic islets as a controlling factor in T1D. First, CXCL10 appears to play a markedly distinct role in the NOD mouse. In the cyclophosphamide-triggered variant of the model, CXCL10 blockade resulted in significant protection, although this was reportedly due

to enhanced β -cell proliferation, while T-cell recruitment to the islets was unaffected (3). β -Cell-inherent effects conferred by CXCL10 were later confirmed by Schulthess and coworkers (4). Contrastingly, however, CXCR3-deficient NOD mice show accelerated diabetes onset (5).

In the RIP-LCMV system, it was shown recently that small-molecule-mediated CXCR3 inhibition was only marginally effective in curbing diabetes onset and progression (6). To reconcile these negative findings with the literature, it was hypothesized that the compound was not sufficiently effective in blocking CXCR3 *in vivo*, although *in vitro* neutralization assays suggested otherwise. It was concluded that the outcome of CXCR3-antagonist administration in the RIP-LCMV model somehow was inferior to treatment with neutralizing antibody to CXCL10 or genetic CXCR3 disruption. The alternative explanation, that the CXCL10/CXCR3 signaling axis is only part of a highly redundant chemokine network rather than a crucial checkpoint, forms the rationale of the current study.

Recent studies demonstrated substantial expression of both CXCL10 and its receptor CXCR3 within islet lesions from T1D patients (4,7–9). Moreover, CXCL10 was upregulated within islets specifically after viral infection, a finding that favors the use of virally induced diabetes models in this context (7). Studies performed within the framework of the network for Pancreatic Organ Donors with Diabetes have revealed, however, that a wide array of chemokines is generally expressed in pancreata from human T1D subjects, which may enable functional redundancy (10). In view of these findings and the re-emerging interest in their translational potential, we systematically evaluated whether the CXCL10/CXCR3 axis is indispensable during T-cell trafficking to islets in a viral mouse model for T1D.

RESEARCH DESIGN AND METHODS

Mice and virus. C57BL/6 (B6), NOD/ShiLtJ, CD45.1⁺ B6.SJL-Ptprca^a Pep3^b/BoyJ, CXCL10-deficient [B6.129S4-Cxcl10^{tm1Adl}/J (11)], and CXCR3-deficient animals [B6.129P2-Cxcr3^{tm1Dgen}/J] mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently bred under specific pathogen-free conditions at our facilities. Transgenic P14 mice expressing a T-cell receptor (TCR) α -chain specific for the H-2D^b-restricted immunodominant LCMV epitope GP33–41 (12) were obtained as a gift from The Scripps Research Institute and maintained in our facilities. For tracking and imaging purposes, we crossed C57BL/6-Tg(ACTB-EGFP)10sb/J (13) mice to the P14 mice. Mice expressing GP antigen from the LCMV-WE (14) or LCMV-Arm (15) strain under control of the RIP (RIP-GP) were bred to a CXCL10- or CXCR3-deficient background and to a strain with β -cell-restricted GFP expression (16) for imaging purposes. All animal procedures were approved by the Animal Care Committee of the La Jolla Institute for Allergy and Immunology (protocol number AP121-MvH3-0510). LCMV Armstrong clone 53b plaques

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Received 3 October 2012 and accepted 17 February 2013.

DOI: 10.2337/db12-1370

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db12-1370/-/DC1>.

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were purified three times on Vero cells, and viral stocks were prepared by a single passage on BHK-1 cells. LCMV strain WE was a gift from Dr. Juan Carlos de la Torre (The Scripps Research Institute). The experiment in Fig. 4I showing CXCL10 neutralization in the RIP-GP model was performed in the Christen laboratory (Frankfurt am Main, Germany) using the same protocol, antibody reagents, and mouse and virus strains for diabetes induction.

LCMV plaque assay. Homogenized spleens from infected animals were incubated at 37°C, 5% CO₂, for 1 h with Vero cell monolayers grown in six-well plates (Costar). The plates were then overlaid with 1% agarose in minimal essential medium 199 (Invitrogen) containing 10% FBS and incubated at 37°C, 5% CO₂, for 5 d. The wells were treated with 25% formaldehyde and stained with 0.1% crystal violet for 2 min. The agarose overlay was removed, and infectious centers were counted. Additionally, viral LCMV stock was used as a positive control.

Diabetes induction protocol. In the viral experiments, diabetes induction was achieved by infection of LCMV.GP-transgenic recipients with 10⁴ plaque-forming units (pfu) LCMV i.p. or 200 pfu LCMV.WE, where indicated. We recently developed a virus-free adoptive transfer model based on the conventional RIP-LCMV.GP system (17,18). Hereto, TCR-transgenic P14 CD8 T cells are transferred into RIP-LCMV.GP host animals followed by peripheral activation using a peptide/adjuvant mixture. Depending on the host strain and in particular its expression of the GP antigen on pancreatic β -cells, all mice develop diabetes in an acute fashion (RIP-LCMV/WE host) or in 50% of mice in a milder form of diabetes (RIP-LCMV/ARM host). Any effects due to specific viral signaling can be excluded in this variant model, and it should be perceived as a model to analyze the capacity of peripherally activated effectors to home to the site of antigen expression in the islets. Briefly, on day 0, a single-cell suspension of 1.5×10^7 P14 splenocytes was transferred into recipient mice via retro-orbital injection. On days 1 and 3, mice were intraperitoneally injected with 2 mg of GP33 peptide (NH₂-KAVYNFATM-COOH; Abgent) in ultrapure water containing 50 μ g of CpG adjuvant (Sigma-Aldrich). Finally, on day 6, mice received 500 μ g of polyinosinic-polycytidylic acid i.p. (Sigma-Aldrich). Blood glucose measurements were performed with a OneTouch blood glucose meter (LifeScan, Inc.). Mice with values >300 mg/dL were considered diabetic unless otherwise indicated. All two-photon imaging was done on days 7 and 8. Glucose tolerance test involved injection of 2 g/kg glucose i.p. into overnight-fasted animals and blood glucose measurements at the indicated time points.

Antibodies for in vivo neutralization. Anti-CXCL10 antibodies for in vivo neutralization were a kind gift from Dr. Andrew D. Luster and were validated previously (19) or purified from hybridoma supernatant (gift from Andrew D. Luster, Harvard Medical School) and tested by an in-house chemotaxis assay. It was administered in an identical fashion as described by Christen et al. (2) (i.e., 100 μ g in 100 μ L of PBS per injection, 6 h before infection and on days 1, 2, 4, and 6 after LCMV infection).

Intracellular cytokine staining assay and flow cytometry. Single-cell suspensions were prepared from spleen and pancreas when indicated. Erythrocyte lysis was performed on splenocyte preparations. NRP-V7 tetramer was obtained from Dr. Rusung Tan (University of British Columbia). For intracellular cytokine staining, cells were restimulated with GP33 antigen for 5 h in the presence of brefeldin A (GolgiPlug; BD Biosciences), stained for surface markers, and subsequently fixed and permeabilized (Cytofix/Cytoperm; BD Biosciences) for intracellular staining. All antibodies were obtained from BD Biosciences. Acquisition was performed on an LSRII flow cytometer (BD Biosciences).

Magnetic bead-assisted cell purification and 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester dilution assay. For experiments presented in Fig. 1, P14 CD8 T cells were purified by depletion prior to transfer using sheep antirat magnetic DYNAL beads (Invitrogen) in conjunction with rat anti-CD4, anti-B220, anti-CD16/CD32, and anti-IA/E antibodies (all from BD Biosciences). Purity was verified by flow cytometry and was typically within the 90–95% range. Purified cells were labeled with 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer's instruction, where indicated (Invitrogen).

Immunohistochemistry and two-photon imaging. Pancreata were harvested and snap-frozen in OCT medium (Sakura), and 6- μ m consecutive frozen sections were cut, acetone-fixed, and stained with anti-CD8a or anti-CD4 and insulin. Immunohistochemical detection was done using biotinylated secondary antibodies and Avidin-D coupled to horseradish peroxidase or alkaline phosphatase, followed by enzymatic development with diaminobenzidine and Vector Blue chromogens (Vector Laboratories). Immunofluorescent quantitation of T-cell islet infiltration was done in analogous fashion except for the use of Alexa Fluor 488-coupled secondaries and Prolong antifade, 4',6'-diamidino-2-phenylindole-enriched mounting medium (both from Invitrogen). Images were acquired on a Nikon Eclipse 80i microscope with fluorescent attachment (Nikon), and islets surface, based on 4',6'-diamidino-2-phenylindole morphology, was calculated using ImageJ (National Institutes of Health). Finally,

a technician unaware of sample identity counted the infiltrating T cells using the Cell Counter plugin.

In vivo two-photon imaging of the pancreas was described in detail in Coppieters et al. (18).

Real-time PCR. Organs were harvested and subjected to RNA isolation using the RNeasy Midi kit (Qiagen), cDNA was prepared using the Quantitect Reverse Transcription kit (Qiagen), and quantitative SYBR Green-based real-time PCR was performed with validated Quantitect primer pairs specific for CXCL10 and 18S ribosomal RNA. Resulting threshold cycle values were used to determine relative expression values using the $\Delta\Delta$ threshold cycle method. Data in Supplementary Fig. 2C used validated CXCL10 and glyceraldehyde-3-phosphate dehydrogenase housekeeping gene primers from SABiosciences.

RESULTS

Systemic CXCL10 or CXCR3 deficiency does not impair viral clearance. Diabetes in the RIP-LCMV.GP diabetes model results from the activation and expansion of T cells in response to LCMV and their subsequent re-direction to the β -cells, which transgenically express the viral glycoprotein (14,15). Analyzing effects on diabetogenicity in this model requires differentiation between effects on activation and expansion during the antiviral phase and on effector recruitment to the islets during the diabetogenic phase. To verify that systemic CXCL10 or CXCR3 deficiency does not interfere with antiviral CD8 T-cell functionality, antigen-stimulated cytokine production was analyzed in gene-deficient mice that were infected 8 days prior (Fig. 1A). Analogous frequencies of antigen-experienced, cytokine-producing cytotoxic T lymphocytes (CTLs) were found as compared with wild-type, indicating that CXCL10 or CXCR3 deficiency does not affect the antiviral response. In agreement with these results, complete viral clearance was consistently achieved (Fig. 1B). Likewise, transferred LCMV-specific CD8 T cells responded similarly to infection in CXCL10-deficient hosts as compared with wild-type (Fig. 1C). Intrinsic CXCR3 deficiency within the transferred CD8 T-cell population did not interfere with activation and expansion of the cells (Fig. 1D). Collectively, these data indicate that neither CXCL10 nor CXCR3 deficiency preclude the development of antiviral CTL immunity in this system and thus validate its use for recruitment studies during the diabetogenic phase.

CXCL10 and CXCR3 upregulation patterns after LCMV infection. It was shown earlier that CXCL10 is upregulated in the pancreas shortly after LCMV infection (2) and that diabetogenic T cells preferably home to sites containing high levels of this chemokine (20). However, quantitative RT-PCR analysis across various organs harvested after LCMV infection revealed even higher expression in kidney, liver, and spleen (Fig. 1E). Thus, LCMV-induced CXCL10 hyperexpression is not a pancreas-restricted response. This observation thus questions the importance of CXCL10 in the specific recruitment of CTL to pancreatic islets.

It was shown previously that LCMV infection results in the expression of CXCR3 on ~93% of activated endogenous GP33–41-specific CD8 T cells (2). In order to confirm CXCR3 expression on TRC-transgenic P14 cells, which are of particular relevance to our transfer model data, we performed a detailed kinetic analysis (Supplementary Fig. 1). The outcome clearly shows that CXCR3 upregulation in the transferred P14 population surpasses the frequencies observed in endogenous CD8 T-cell responders after viral infection. These data therefore indicate that the transferred cells should be responsive to CXCL10-mediated chemotaxis and validate the use of the transfer model for our purposes.

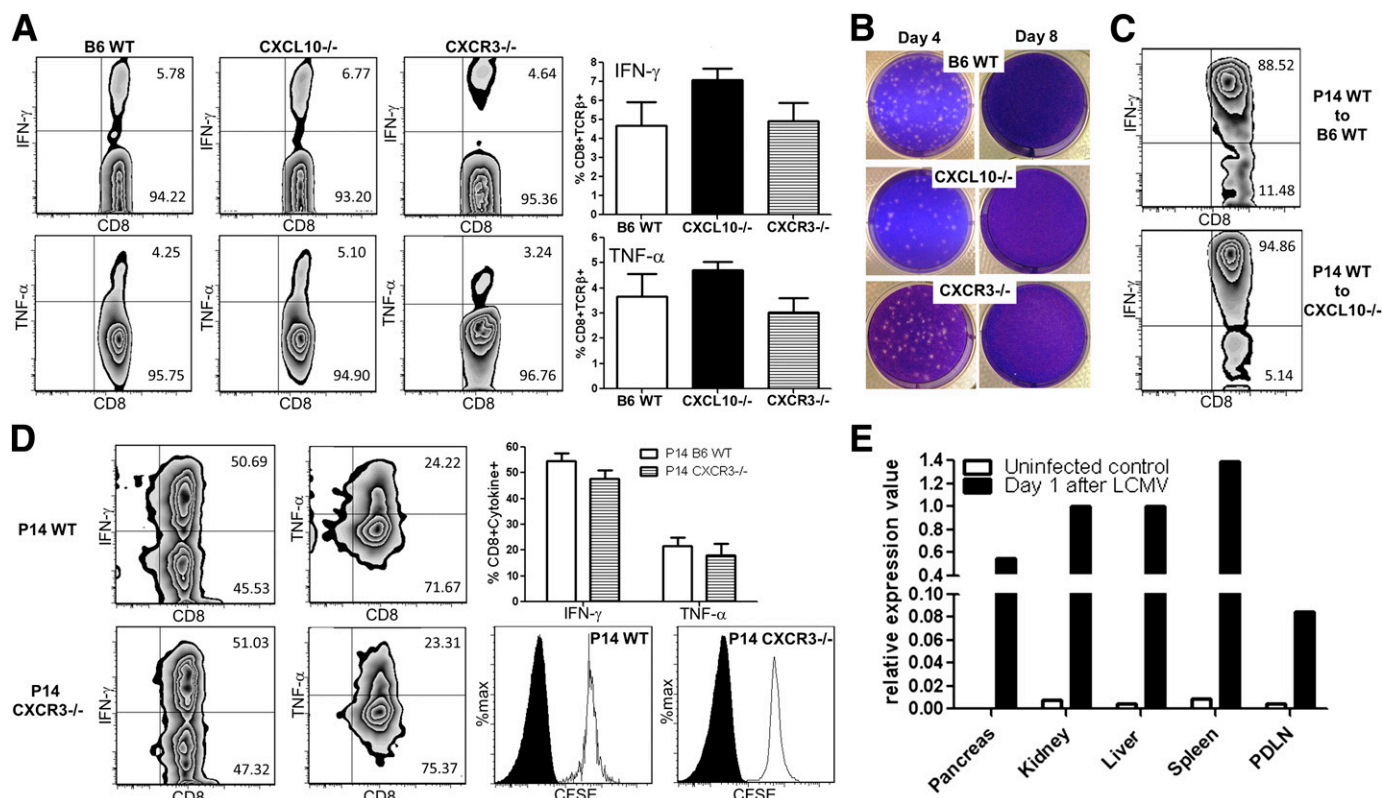


FIG. 1. Normal expansion and functionality of antiviral CD8 T-cell effectors in the absence of CXCL10 or CXCR3 signaling during LCMV Armstrong infection. **A:** Wild-type (WT), CXCL10^{-/-}, and CXCR3^{-/-} animals were infected with LCMV, splenocytes were harvested 7 days later, and GP33 antigen-stimulated responses were measured by intracellular cytokine staining (ICCS). Gated on CD8⁺TCR β ⁺ cells. Experiment was performed in triplicate. **B:** WT, CXCL10^{-/-}, and CXCR3^{-/-} mice were infected with LCMV and viral loads (pfu/g) were determined in the spleen on days 4 and 8 after infection. On day 4, respective loads were 15×10^4 pfu/g (WT), 8.6×10^4 pfu/g (CXCL10^{-/-}), and 11×10^4 pfu/g (CXCR3^{-/-}). Experiment was performed in duplicate. **C:** WT and CXCL10^{-/-} recipients were supplemented with 10^5 purified, GFP-labeled P14 CD8 T cells and infected with LCMV 1 day after transfer. On day 8, spleens were harvested, and ICCS was performed for interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) after GP33 antigen stimulation. Gated on GFP⁺CD8⁺TCR β ⁺ cells. Experiment was performed in triplicate. Naive WT TCR-transgenic splenocytes were included as controls and showed some baseline production for TNF- α but not IFN- γ (1.69% double positive [DP]), indicating that IFN- γ production is better suited to identify antigen-experienced effectors among the transferred cells. **D:** The effect of CXCR3 deficiency specifically on CD8 T cells was analyzed by transferring 10^5 purified, CD45.2⁺ CFSE-labeled WT or CXCR3^{-/-} P14 CD8 T cells into CD45.1⁺ congenic recipients. Eight days after LCMV infection, CD45.2⁺ cells were identified in the spleen, CFSE dilution was measured, and ICCS performed after GP33 antigen stimulation. Experiment was done in triplicate, and plots are TCR β ⁺ gated. Filled CFSE plots represent infected versus open, which is uninfected control. **E:** Organs were harvested from naive animals 1 day after LCMV infection and subjected to quantitative real-time PCR for CXCL10. PDLN, pancreatic draining lymph node. (A high-quality color representation of this figure is available in the online issue.)

In view of published reports (21), we also performed parallel analyses for CCR5 and CCR2 expression across different organs during the development of diabetes in the RIP-LCMV model and the related transfer model (Supplementary Fig. 1). Our data show that CXCR3 expression on the dominant diabetogenic CD8 T-cell population is clearly highest and increases over time in all compartments including the pancreas. CCR2 expression is close to undetectable, whereas CCR5 expression significantly increases, particularly in the pancreas. The finding that CCR5 is overexpressed in the diabetic pancreas in our model is in line with NOD data by Carvalho-Pinto et al. (21), although effector influx per se was not affected in that paper. Of note, we tried to block the CCR5 ligand CCL5 (regulated on activation, normal T cell expressed and secreted) with a neutralizing antibody before in the RIP-LCMV model but found no effect (2). More recent data using CCL5-knockout mice indicate that there is no difference in viral clearance, T1D incidence or islet infiltration (S.L., E.H., U.C., unpublished observations).

We next evaluated whether CXCR3 expression is also present on spontaneously arising, low-affinity diabetogenic

CD8 T cells (Supplementary Fig. 2A and B). Hereto, we used the NRP-V7 clone, which is known to react against the islet antigen IGRP in NOD mice in which this clone constitutes a major effector population (22). From our studies, it is clear that around diabetes development, the CXCR3 expression on these low-affinity diabetogenic clones upregulate CXCR3 as diabetes progresses and, in this regard, mirror the P14 cells used in our present report. We also verified that the CXCR3-binding chemokine of interest, CXCL10, is expressed in the pancreas of NOD mice (Supplementary Fig. 2C). We thus conclude that both ligands (in the target organ) and receptors (on low-affinity effectors) are present within the NOD system, yet a lack of CXCR3 does not confer protection in this model (5). We therefore conclude that it is unlikely that the degree of dependence on CXCR3/CXCL10 signaling required for effector influx into pancreatic islets differs between high- and low-affinity T-cell clones in these diabetes models. Similar data as in the RIP-LCMV model were also obtained with regard to the relative levels of CXCR3 expression versus CCR5 and CCR2 (Supplementary Fig. 2A and B).

Modest protection from diabetes development in the absence of CXCR3. Our initial *in vivo* data under conditions of CXCR3 deficiency suggested that the receptor is not required for diabetes development (Fig. 2A). These data were obtained after intraperitoneal infection with 10^4 pfu of LCMV, as commonly used in our laboratory. Immunohistochemical staining for CD8 and CD4 further revealed that T-cell recruitment to pancreatic islets was unaffected (Fig. 2B and C).

As we specifically aimed to replicate the dataset obtained by Frigerio et al. (1), we altered our model by performing intravenous infection with 200 pfu of LCMV-WE instead of the LCMV-Armstrong strain. In contrast to their report, we did not observe a potent suppression of >2 weeks, yet a statistically significant delay could be observed (Fig. 2D). This delay was more prominent when

a conventional hyperglycemia limit was used (300 mg/dL; $P = 0.003$; Fig. 2D, top panel) rather than the limit used by Frigerio et al. (1) (upper meter limit of 600 mg/dL; Fig. 2D, bottom panel), which only exhibits borderline significance in our studies ($P = 0.04$). Our work also shows that >90% of mice eventually become diabetic, regardless of CXCR3 deficiency. We conclude that ablation of CXCR3 does not protect from diabetes development yet offers a modest delay in diabetes progression in the RIP-LCMV model.

Finally, we exploited a recently described virus-free transfer model to study the role of CXCR3 specifically on transferred diabetogenic T-cell effectors (17,18). In brief, naive effector P14 CD8 T cells are activated and expanded *in vivo* by antigen administration in conjunction with Toll-like receptor–ligating adjuvants and subsequently home to the islets of the RIP-LCMV host. Depending on the

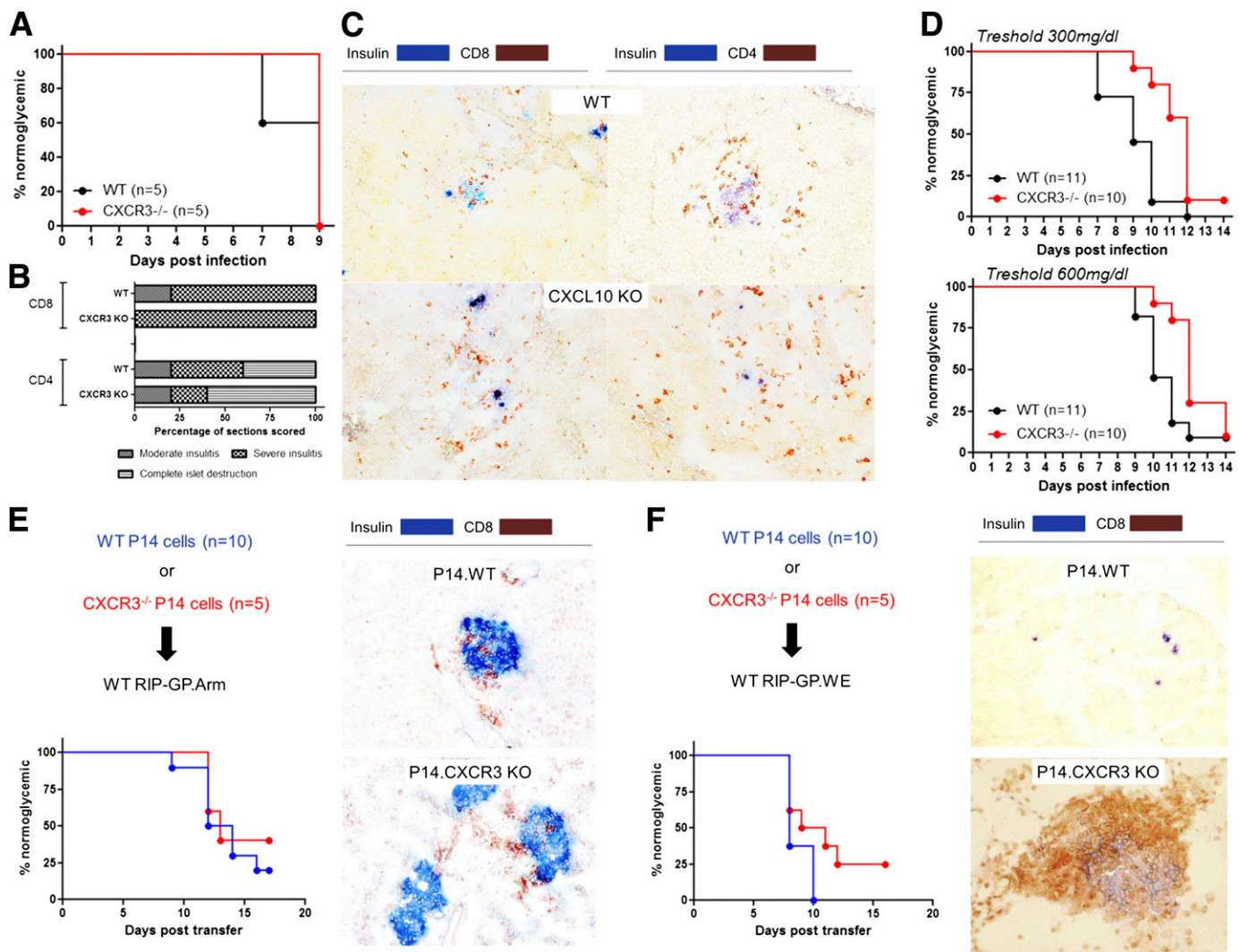


FIG. 2. The CXCR3 receptor has a limited role in T-cell recruitment or diabetes progression in the RIP-LCMV.GP system. **A:** Age- and sex-matched wild-type (WT) and CXCR3^{-/-} RIP-LCMV.GP animals were infected with LCMV.Arm and blood glucose values were measured. Hyperglycemia was defined as >300 mg/dL. **B:** Animals from the study depicted in A were killed at the day of hyperglycemia development, and pancreas tissue was harvested for immunohistochemistry. Insulinitis scores were obtained in a blinded fashion. **C:** Representative images from animals included in panel B show quantitatively equal CD4 and CD8 infiltration in remaining islets at the day of hyperglycemia development. **D:** Analogous experiment as in A but using 200 pfu of LCMV.WE *i.v.* [as in Ref. 1]. Thresholds of 300 and 600 mg/dL were used for optimal comparison with data from Frigerio et al. (1). **E:** CXCR3-deficient or WT P14 CD8 T cells were transferred to WT RIP-GP.Arm recipients (incomplete incidence model) and activated *in vivo* with peptide and adjuvant as described in RESEARCH DESIGN AND METHODS. Blood glucose was monitored and histology performed at conclusion of the experiment. **F:** Experiment was performed in analogous fashion as described for E but with the use of RIP-GP.WE recipients (~100% incidence). While there was a small, nonsignificant trend toward protection, histology and flow cytometric analysis (data not shown) revealed that even nondiabetic CXCR3 deficient animals had high degrees of CD8 T-cell infiltration in the islets. KO, knockout.

background of the host, the model is more or less acute and penetrant (17). It was also confirmed in this study that major histocompatibility complex class I is upregulated in the transfer model, which is considered essential for CD8 T cell-mediated β -cell killing (Supplementary Fig. 3). In line with the data from the conventional viral model, we found that CXCR3-deficient CD8 T-cell effectors provoked hyperglycemia with a marginally reduced efficiency, regardless of host background (Fig. 2E and F). Histology data confirmed that CD8 T-cell migration to the islets was generally comparable to controls. We conclude that T cell-intrinsic CXCR3 expression is not required for diabetes development in this model yet modestly influences the course of disease progression.

No significant effect of systemic CXCL10 deletion or antibody neutralization on diabetes development. Diabetes onset and progression in CXCL10-deficient RIP-LCMV mice was identical as in wild-type mice (Fig. 3A). Detailed histological analysis indicated that T-cell recruitment to the pancreatic islets was entirely preserved (Fig. 3B and C). In agreement with this finding, data using CXCL10-deficient hosts in the transfer model described above showed no significant differences (Fig. 3D). Next, we transferred labeled TCR-transgenic CD8 T cells into CXCL10-deficient RIP-LCMV mice and analyzed pancreatic recruitment after LCMV infection by two-photon microscopy as recently reported (23) (Fig. 4). In correspondence with our histology data, it was found that the ability of the effectors to extravasate and migrate through the pancreas tissue remains intact under conditions of CXCL10 deficiency.

Since these data contradicted the important role previously ascribed to CXCL10 in CD8 T-cell homing, we questioned whether timed neutralization, rather than systemic ablation, could indeed mediate protection. The first experiment was performed with CXCL10-neutralizing antibody donated by Dr. Andrew D. Luster (Harvard Medical School), the clone previously assessed in the RIP-LCMV model (2) (Fig. 5A). In this paper, a nonsignificant tendency toward acceleration was noted in the treated

animals as compared with control. To evaluate the role on T-cell homing to the islets, we performed immunohistochemical (Fig. 5B and C) and immunofluorescent staining followed by quantification of CD8⁺ and CD4⁺ cell numbers within islets (Fig. 5D–F), which indicated no effect on T-cell accumulation. Normoglycemic mice were subjected to glucose tolerance testing, but no differences were observed (Fig. 5G).

Finally, two independent experiments were simultaneously conducted using the same antibody clone, freshly purified from hybridoma supernatant that was tested in our laboratory for complete blockade of CXCL10-mediated chemotaxis *in vitro*. The experiment performed in the von Herrath laboratory demonstrated no effect whatsoever (Fig. 5H), whereas the experiment in the Christen laboratory showed a tendency toward deceleration of diabetes development ($P = 0.0504$; Fig. 5I).

DISCUSSION

The present series of preclinical experiments was designed to assess whether CXCL10/CXCR3 signaling truly represents a key checkpoint in the recruitment of T cells to pancreatic islets in T1D. Our data reveal that neither chemokine nor receptor is required for full antiviral responsiveness and viral clearance in the LCMV system. In combination with the fact that CXCL10 and CXCR3 have been linked to the development of diabetes development in virus-infected patients (7), the RIP-LCMV viral system therefore appears adequate to study the role of this chemokine axis in T-cell recruitment during autoimmune diabetes.

Two publications previously ascribed a pivotal role to CXCL10/CXCR3 signaling in the RIP-LCMV diabetes model (1,2). Frigerio and coworkers (1) observed a substantial delay in diabetes development in the absence of CXCR3. However, an unconventionally high diabetes threshold was used (600 mg/dL vs. conventional 250–300), and it is uncertain what the clinical correlate thereof is. Our data now indicate that the effect of CXCR3 deficiency exists but is

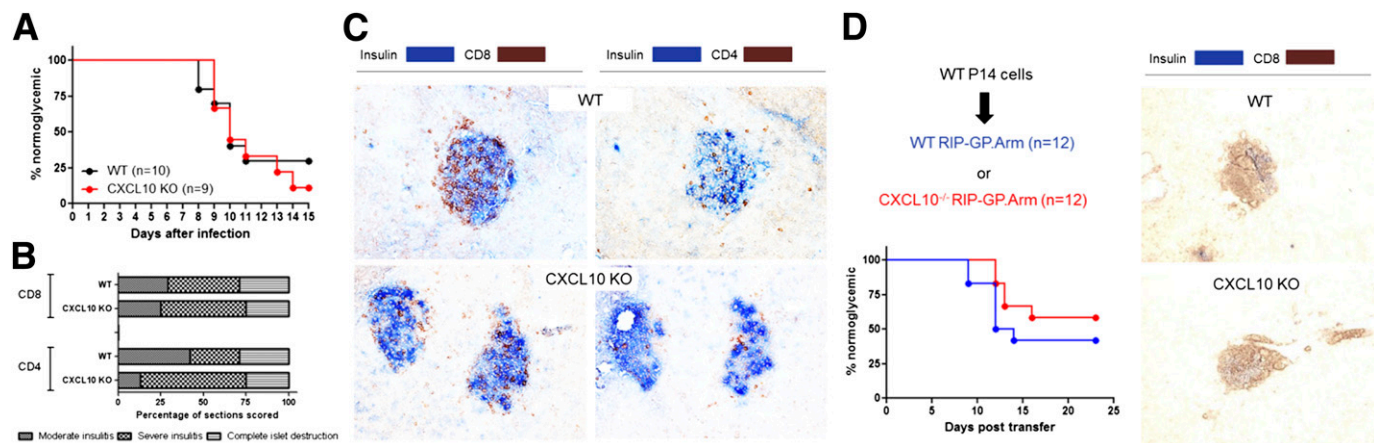


FIG. 3. CXCL10 ablation does not significantly affect T-cell recruitment or diabetes progression in the RIP-LCMV.GP system. **A:** Age- and sex-matched wild-type (WT) and CXCL10^{-/-} RIP-LCMV.GP animals were infected with LCMV, and blood glucose values were measured. Hyperglycemia was defined as >300 mg/dL. **B:** Animals from the study depicted in **A** were killed at the day of hyperglycemia development, and pancreas tissue was harvested for immunohistochemistry. Insulinitis scores were obtained in a blinded fashion. **C:** Representative images from animals included in panel **B** show quantitatively equal CD4 and CD8 infiltration in remnant islets at the day of hyperglycemia development. **D:** WT P14 CD8 T cells were transferred to CXCL10^{-/-} RIP-GP.Arm mice (incomplete incidence model) and activated *in vivo* with peptide and adjuvant as described in RESEARCH DESIGN AND METHODS. Blood glucose measurements were recorded, and pancreas histology was performed after conclusion of the experiment. KO, knockout.

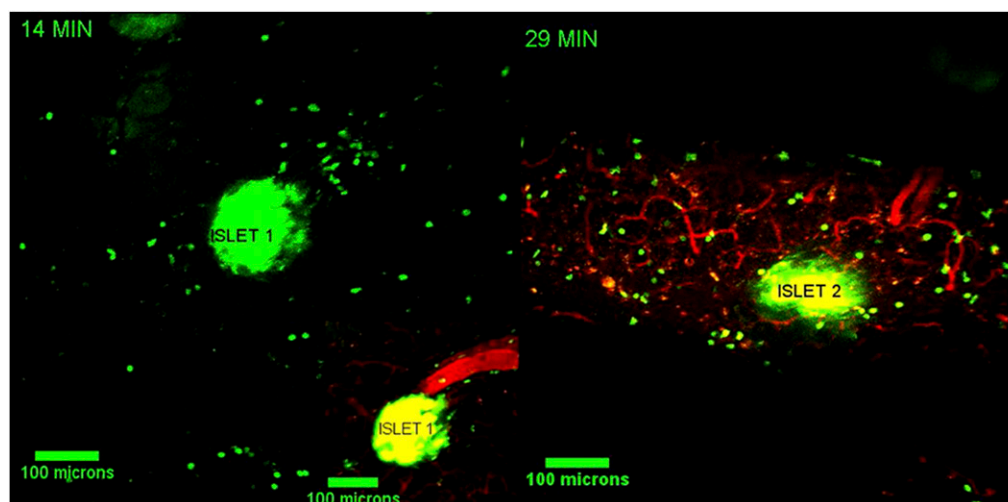


FIG. 4. Virally expanded, diabetogenic CD8 T cells efficiently migrate to the pancreatic islets in vivo in the absence of CXCL10 signaling. Figure shows two panels of different pancreatic regions that are part of 14- and 29-min time-lapse sequences displaying two individual islets from CXCL10-deficient animals as captured by in vivo two-photon imaging. Purified GFP-labeled P14 cells were transferred to RIP-GP animals expressing GFP under the insulin promoter. Eight days later, mice were subjected to imaging, and islets and infiltrating CD8 T cells are both visible in green. The vasculature was then stained in red by injection of a vascular dye. Note the high frequency of extravasated P14 cells, indicating that these effectors are capable of migrating to the exocrine pancreas and islets under conditions of CXCL10 deficiency. Acquisition is a maximum intensity projection series and consists of images spanning 25 z-steps spaced 5 μm apart at a 1-min time interval. Representative of imaging data obtained from three individual mice.

rather subtle and depends on factors such as route of infection, viral strain, and dose. By no means do our experiments indicate that CXCR3 is essential for diabetes development or T-cell recruitment in this model, although a certain contribution does seem plausible. These findings are entirely in agreement with recent data using small-molecule CXCR3 inhibitors in the RIP-LCMV model, which also showed marginal efficacy (6).

Of note, conventional B6 mice lack a functional *Cxcl11* gene, another CXCR3 ligand with higher affinity for the CXCR3 receptor than CXCL10 and CXCL9 (24). We checked the history of B6.129S4-Cxcl10tm1Adl/J and B6.129P2-Cxcr3tm1Dgen/J mice, and, as with our RIP-LCMV mice, these animals were backcrossed for numerous generations to the conventional B6 genetic background. It is therefore probable that these strains lack functional CXCL11. Our earlier study in the RIP-LCMV model (2) indeed found very small amounts of CXCL11 transcript in the pancreas. The potential presence of functional CXCL11 in the (C57BL/6 $\times$ BALB/c) F1 mice used in the study by Frigerio et al. (1) could theoretically account for the difference in diabetes protection between our respective CXCR3-deficient RIP-LCMV strains. However, Frigerio et al. (1) reported that they "barely detected specific transcripts for this factor (CXCL11) in insulinitic islets using quantitative PCR." We can therefore conclude that it is unlikely that this potential strain-specific difference in CXCL11 expression will account for our divergent findings.

The current study investigated the issue from several different angles, using CXCL10 (never reported) and CXCR3-deficient [as in Frigerio et al. (1) but on a clean B6 background] RIP-LCMV mice to evaluate the impact of genetic deletion. A new virus-free transfer model was used to test the role of T cell-intrinsic CXCR3 signaling, while antibody-mediated CXCL10 treatment was performed to investigate the effect of timed chemokine neutralization. Thus, the present paper offers a thorough reassessment of the true importance of CXCR3/CXCL10 signaling in the RIP-LCMV system and provides conclusive evidence that

therapeutic blockade of this axis does not curb autoimmune diabetes as previously reported. This reconciles negative findings from the small-molecule inhibitor study as well as data by others in the NOD model and conveys the important message that antichemokine monotherapy may not offer therapeutic benefit, likely due to the high degree of redundancy.

With regard to CXCL10, previous results using antibody-mediated blockade suggested that >70% of mice may experience lasting protection (2). In an elaborate set of experiments, we now find that the therapeutic potential of CXCL10 inhibition is in fact rather limited, if existent. This finding is corroborated by the generation of a CXCL10-deficient RIP-LCMV strain that showed unaltered diabetes development. The cause for these conflicting antibody neutralization data are not clear, but the problem might potentially lie in the antibody batch used at the time, which for instance might have been contaminated or cross-reactive. We confirmed that the present antibody batch neutralized well in vitro but obviously cannot directly compare with the potency of the batch used >10 years ago. Interestingly, in some experiments, a tendency toward protection was noted while others showed almost matching incidence curves. We infer that laboratory-specific differences in mouse background, vivarium conditions, and viral stocks may account for the lack of a consistently penetrant, minor tendency toward protection. The overarching message, however, is that the effect of CXCR3 or CXCL10 ablation/inhibition is much more subtle in this model than previously assumed.

While CXCL10/CXCR3 signaling may well be essential in *in vitro* chemotaxis assays, we question whether the abundant, nonexclusive expression of chemokines across the entire organism in response to viral infection critically governs the specific recruitment of T cells to pancreatic islets in vivo. Indeed, we find in this study that upregulation of CXCL10 is not a pancreas-specific phenomenon during disease development. Furthermore, Sarkar et al.

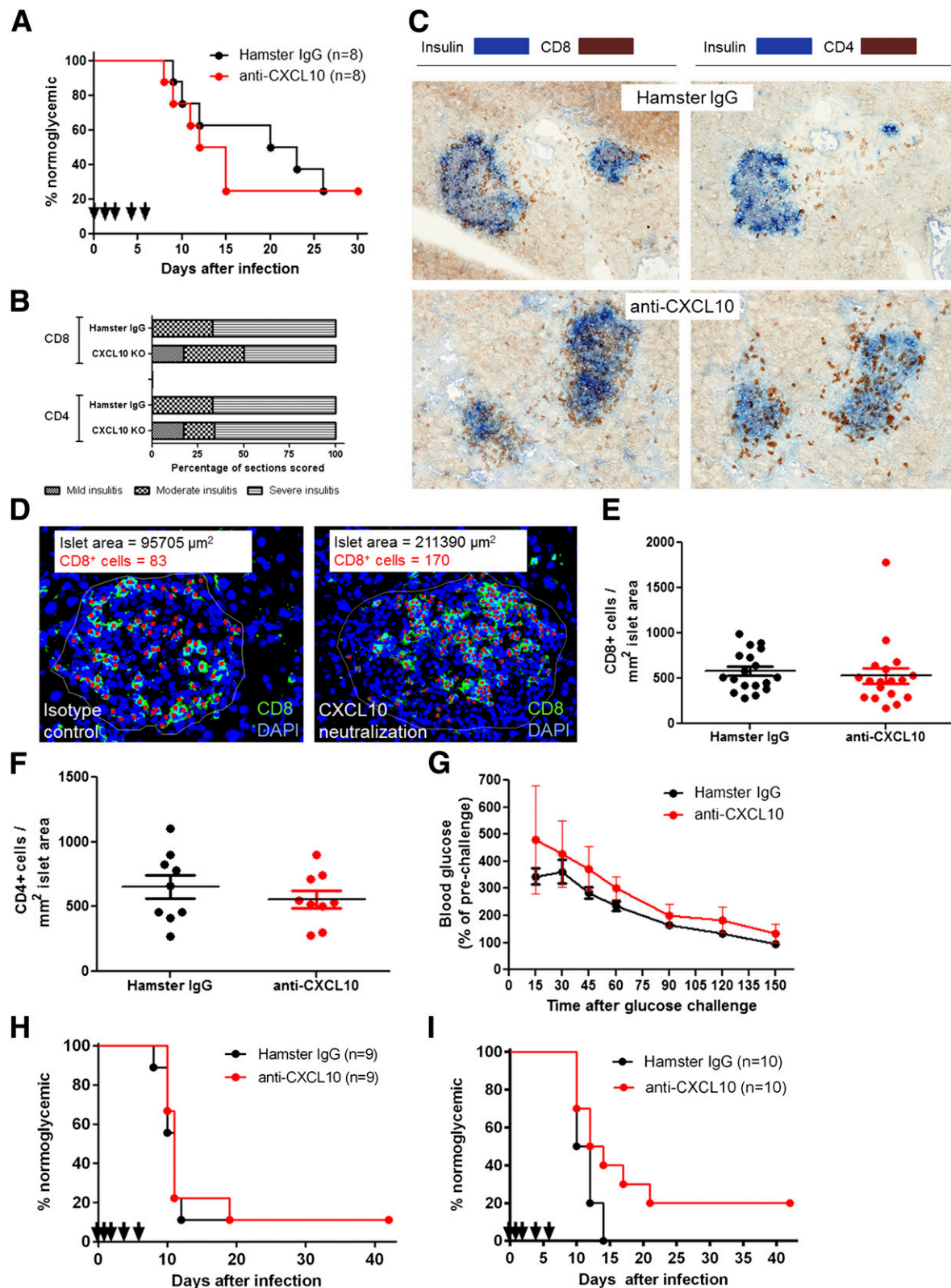


FIG. 5. Therapeutic CXCL10 blockade does not significantly affect T-cell recruitment or diabetes progression in the RIP-LCMV.GP system. **A:** Diabetes incidence upon antibody-mediated CXCL10 blockade (gift from Andrew D. Luster, Harvard Medical School). Timing of injection (100 μg dose; arrows) was on days 0 (–6 hours), 1, 2, 4, and 6. **B** and **C:** Histological assessment revealed no significant differences in T-cell recruitment to the pancreatic islets. **D–F:** Pancreata from diabetic mice in **A** were sampled and stained for CD8 and CD4 (**D**, green; representative images shown for CD8 only), infiltrating numbers were counted using ImageJ (**E** and **F**, red dots mark counted cells; National Institutes of Health), normalized to islet surface (white line), and values plotted. **G:** The two nondiabetic animals from each treatment group in **A** were subjected to a glucose tolerance test and displayed similar responses. **H** and **I:** Identical experimental setup as in **A**, but using freshly prepared antibody from the same hybridoma as in **A** (Christen laboratory; verified in chemotaxis assay). Experiment in **H** was performed in La Jolla, CA (by M.G.v.H.), while in **I**, it was performed simultaneously in Germany (by U.C.). Arrows on x-axis indicate timing of antibody injections. DAPI, 4',6'-diamidino-2-phenylindole; IgG, immunoglobulin G; KO, knockout.

(10) recently showed that CXCL10 expression is not restricted to islet lesions in mice and human T1D patients, but is generally expressed across both endocrine and exocrine tissue. It is thus unlikely that β -cells are specifically responsible for the recruitment of CD8 T-cell effectors to pancreatic islets in vivo, as suggested by Frigerio et al. (1). This hypothesis was also tested by Rhode and colleagues (25), who created RIP-CXCL10 mice, which express massive amounts of the chemokine specifically in their pancreatic islets and show spontaneous insulinitis and aggravated diabetes. It can thus be assumed that non-physiological overexpression of CXCL10 results in its domination over other factors and accelerated pathogenesis, while during the natural disease course of T1D, other factors might be at least equally important.

Finally, in the cyclophosphamide-induced NOD mouse model, signaling is not required for the recruitment of diabetogenic effectors (3). Ablation of CXCR3 even leads to acceleration of diabetes in the NOD model system (5). Further supporting this notion are recent data demonstrating that prolonged, systemic CXCR3 blockade does not affect T-cell recruitment and diabetes development in the RIP-LCMV model (6). It can thus be inferred that there is no universal role for this chemokine axis in T-cell trafficking to pancreatic islets.

In conclusion, our present results challenge the notion that the CXCL10/CXCR3 chemokine axis is a potential target for therapeutic neutralization in recent-onset T1D. We argue that blockade of a single chemokine may be insufficient to abrogate T-cell recruitment to islets and prevent β -cell decay.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health/National Institute of Allergy and Infectious Diseases Grant R01-DK-66346-05 (to M.G.v.H.).

K.T.C. and M.G.v.H. are currently employed by Novo Nordisk. No other potential conflicts of interest relevant to this article were reported.

K.T.C. performed and designed experiments, interpreted data, and wrote the manuscript. N.A., P.P.P., C.B.J., A.W., S.L., and E.H. performed experiments. U.C. designed experiments and interpreted data. M.G.v.H. designed experiments, interpreted data, and wrote the manuscript. M.G.v.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Mira Bel Hani and Yulia Manenkova (both of the La Jolla Institute for Allergy and Immunology) for technical assistance during revision of the manuscript. The authors also thank Dr. Andrew D. Luster (Massachusetts General Hospital, Harvard Medical School) for the donation of anti-CXCL10 antibody and Carol P. Leary (Massachusetts General Hospital, Harvard Medical School) for coordination of preparation and shipping.

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